Growth and lipid levels of *Tetraselmis tetrahele* and *Nannochloropsis* sp. cultured under commercial fertilizers

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ABSTRACT

Microalgae are aquatic photosynthetic organisms that contain high amounts of lipid and are potential sources of biofuels as well as feed additives for aquaculture. This study analyzed the growth and algal lipid content of two microalgae species (*Tetraselmis tetrahele* and *Nannochloropsis* sp.) using commercial fertilizers and nutrient enrichment. The samples were cultured for 5 days in 1 L dextrose bottles fertilized using Tongkang Marine Research Laboratory (TMRL) enrichment media, inorganic fertilizers such as 14-14-14, and a combination of 14-14-14 and 21-0-0. The relative growth rate of two algae were measured by computing the *k* value while the lipid components were extracted using the Bligh and Dyer Method, and the lipid content of each sample was determined using the gravimetric method. The use of 14-14-14 fertilizer produced the highest growth rates (k=1.810) and lipid composition (14.789%) for *T. tetrahele*. By contrast, *Nannochloropsis* sp., grew well under TMRL enrichment media (k=9.708), and the use of 14-14-14 fertilizer resulted to high lipid content (5.000%).

Keywords: lipid content, microalgae, biofuel, nitrogen, aquaculture

INTRODUCTION

Microalgae are aquatic photosynthetic organisms with different sizes ranging between 1 μm and 2 mm (Baharuddin et al. 2016). Studies on microalgae gained global attention recently due to its numerous usage and application in the area of energy production, pharmaceuticals, food production, aquaculture, and waste recycling. Some studies published include the important role of microalgae in solubilizing high amount of CO_2 (Takagi et al. 2006), bioaccumulation of heavy metals (Naorbe and Serrano 2018), and feed ingredients in aquaculture (Seong et al. 2019).

Microalgal biomass is mostly composed of lipids, carbohydrates, and proteins (Choudhary et al. 2015). An increase in the lipid content of microalgae has an effect on the amount of protein and carbohydrates present. The quantities of microalgal lipids vary with the species, growth conditions, and the culture environment. Furthermore, the biomass of microalgae is correlated to the amount of lipid that could be extracted (Mata et al. 2010).

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The high amount of lipid in microalgae is one of the reasons why they are potential sources of biofuels (Singh and Gu 2010; Maizatul et al. 2017). Land-based crops are also considered as alternative sources but they are also used as primary sources of food and require large areas of land for production hence the preference on microalgae for biofuel production (Bajpai and Tyagi 2006; Maruyama et al. 2009; Maizatul et al. 2017).

Microalgae are more efficient converters of solar energy due to their simpler cellular structure compared to land-based crops (Mata et al. 2010; Griffiths et al. 2011). Other favorable characteristics include the relatively low cost associated with harvesting and transportation compared to those of other biomass materials such as trees and other crops. Also, by virtue of their relatively small sizes, microalgae can be chemically treated easily and can be grown under conditions which are deemed unsuitable for conventional crop production. These characteristics of microalgae enable the production of up to 30 times the amount of oil per unit area of land compared to land-based oilseed crops (Chisti 2007; Mata et al. 2010; Griffiths et al. 2011).

In the field of aquaculture, microalgae serve as food and feed additives during the early larval stages of shrimps, mollusk, echinoderms and other fishes, thus considered essential in aquaculture (Han et al. 2019).

The problems associated with the production of biofuels from algae and algal production for aquaculture in general is that it cannot compete in the global market due to high production cost, labor-intensive requirement, algal crashes, and contamination (Alabi et al. 2009; Leite et al. 2013). Algae-based biofuel and feed additives can be commercialized and be produced in a much larger scale with the development of a suitable cost-effective growth medium, low-energy intensive harvesting method, and an effective lipid extraction method.

Several studies on increasing the production of lipids in microalgae and suitable lipid extraction methods have been published in the past few years. The focus is on minimizing production cost through alternative culture systems such as ponds, raceways, and canals as well as using cheap and readily-available sources of nutrients such as wastewater (Nzayisenga et al. 2020). The findings suggest that environmental condition and nutrient starvation can alter the lipid content in microalgae (Zhu et al. 2016), and that there is a need to identify suitable algal strains to be used for the production of lipids (Venteris et al. 2014).

To contribute to the research for possible strains of microalgae with potential for mass production, this study analyzed the growth and lipid content of *T. tetrahele* and *Nannochloropsis* sp. when cultured using

commercial fertilizer in an indoor setup. This study gives information on growth and the amount of lipid present in the two microalgae species.

METHODS

Experimental Design

Two experiments were simultaneously conducted and each experiment was subjected to three treatments with three replications (Table 1). There were two variables in this study namely: independent - the two microalgae cultured using nutrient enrichment and commercial fertilizers; dependent - the growth rate and lipid content of the microalgae.

In these experiments, two microalgal species namely: *T. tetrahele* and *Nannochloropsis* sp. were selected. Both of these species are widely used in aquaculture and were reported to contain high amount of lipids which could potentially be used in the production of biodiesel. These algal species were sourced from pure starter cultures of the Phycology Laboratory of the College of Fisheries and Ocean Sciences (CFOS) at the University of the Philippines in the Visayas (UPV), Miagao, Iloilo. The experiment was conducted at the Wet Laboratory of the aforementioned college.

Table 1. Experimental design with estimated nitrogen-phosphorus-potassium (N-P-K %) and initial cell density. R – replications. *in the form of potassium nitrite and sodium ortho phosphate.

Treatments	R	Percent Nutrients			Experiment Initial Density (cells per ml)	
		N	*P*	*K*	Experiment 1 *Tetraselmis tetrahele*	Experiment 2 *Nannochloropsis* sp.
TMRL enrichment media	3	*	*	*	1,070,000	8,500,000
14-14-14	3	14	14	14	1,070,000	8,500,000
Combination of 14-14-14 and 21-0-0 fertilizer (1:2)	3	35	14	14	1,070,000	8,500,000

Algal Cultures

The 18 dextrose bottles were sterilized prior to use in the experiment in order to eliminate potential bio-contaminants. Likewise, the hoses and pipes used for aerating the cultures were also sterilized. The seawater used in

the culture has a salinity of 35 ppt and this was chlorinated, dechlorinated, and stocked for 24 hours before using. The initial density of the algae used in both experiments are stated in Table 1. The initial and final densities of *T. tetrahele* and *Nannochloropsis* sp. were determined using a haemacytometer (corner blocks for *T. tetrahele* and center squares and corner blocks for *Nannochloropsis* sp.) under an electron microscope at 10x magnification. Samples of *T. tetrahele* were treated with Lugol's iodine solution in order to immobilize the motile cells prior to counting.

The enrichment media Tongkang Marine Research Laboratory (TMRL) and treatments using commercial fertilizers were prepared as follows; TMRL was prepared based on the standard composition of TMRL medium containing 100 g of potassium nitrite, 10 g of sodium orthophosphate, 3 g of ferric chloride and 2 g of sodium silicate dissolved in 1000 ml of distilled water; 14-14-14 – 50 g of 14-14-14 dissolved in 1000 ml of distilled water and combination – 50 g of 14-14-14 and 100 g of 21-0-0 with 1:2 ration dissolved in 1,000 ml distilled water.

All of the 1 L dextrose bottles were at first filled with 800 ml of treated seawater followed by the addition 1 ml prepared fertilizers/nutrient media and finally with the 200 ml of inoculum. The dextrose bottles were distributed randomly in an indoor shelf equipped with fluorescent lights to support the growth of microalgae. Water temperature ranges from 26-28^{o}C and each bottle is provided with constant aeration for six days (Figure 1).

Growth Measurement

To determine the relative growth rate *(k* value*)* was calculated using Guillard's (1973) formula as described by Ronquillo et al. (1997):

$$k^1 = \frac{log_2\left(\frac{N_1}{N_0}\right)}{t_1 - t_2}$$

Where k = number of divisions per day; N_1 = concentration of cells in the culture at t_1; N_o = concentration of cells at t_o; t_1 = the final culture time in days; and t_o = the time initial cultur, e time in days. The mean *k* values and standard errors of the mean (SEM) were computed.

Figure 1. Indoor algal production in 1 L dextrose bottles at the Phycology Laboratory of the College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo.

Lipid Analysis and Lipid Extraction

Dry method and Bligh and Dyer method of extracting lipids were performed for lipid analysis. The dry method was done by washing the 10 ml *T. tetrahele* and *Nannochloropsis* sp. with distilled water twice after the centrifuge process (200 rpm). Prior to the experiment for dry weight and Bligh and Dyer method, the crucibles were oven dried and placed inside the desiccator to remove moisture. Lipid extraction was carried out by using 2:1 MeOH (methanol:chloroform). The extracted lipids from the algae were placed in the crucible and was oven dried for 3 h at 50°C. After drying, the samples were placed in the desiccator for moisture removal. After an hour, the final weight of the crucibles was measured using an analytical balance.

Data Analyses

The lipid content of the two microalgae species was analyzed using the approximate gravimetric method by Bligh and Dyer (1959). The extraction and partitioning methods were simultaneous and the precipitated proteins were isolated between the two liquid phases (Bligh and Dyer 1959). This method is particularly suitable for the lipid extraction of incubation medium, tissue homogenates, or cell suspensions. Each replicate was sampled five times for a more accurate statistical evaluation.

The one-way ANOVA with Tukey's test for multiple comparison at $p<0.01$ were carried out to compute for statistical difference in terms of growth rate and extracted lipids within the same species of microalgae.

RESULTS

Growth Rate

The relative growth rates (*k*) of *T. tetrahele* and *Nannochloropsis* sp. are shown in Table 2. The growth rates of microalgae in each treatment were significantly different from each other depending on the species and treatments they were exposed with (Table 2). *Tetraselmis tetrahele*, cultured under 14-14-14 had the highest *k* value of 1.810±0.36 while *Nannochloropsis* sp. cultured under TMRL had a significantly high *k* value which is 9.708±0.44 (Table 2).

Table 2. Average (±SEM) *k* values of *Tetraselmis tetrahele* and *Nannochloropsis* sp. exposed to different nutrients (n=5).

Nutrients	***Tetraselmis tetrahele*** **Mean (±SEM) *k* value (cell/day^{-1})**	***Nannochloropsis* sp. Mean (±SEM) *k* value (cell/day^{-1})**
TMRL	0.143±0.21^{c}	9.708±0.44^{a}
14-14-14	1.810±0.36^{a}	0.721±0.23^{b}
Combination of 14-14-14 and 21-0-0 fertilizer	0.860±0.07^{b}	0.816±0.11^{b}

Average Lipid content

The results showed that *T. tetrahele* had higher average lipid content than *Nannochloropsis* sp. in all three types of nutrients (Figure 2). *Tetraselmis tetrahele* cultured under 14-14-14 (14.789%) had a significantly high ($p<0.01$) lipid content than those cultured under the combination and TMRL (Figure 2). Similar results for *Nannochloropsis* sp. were observed wherein those cultured under 14-14-14 had a significantly ($p<0.01$) high lipid content of (5%) compared to other two treatments (Figure 2).

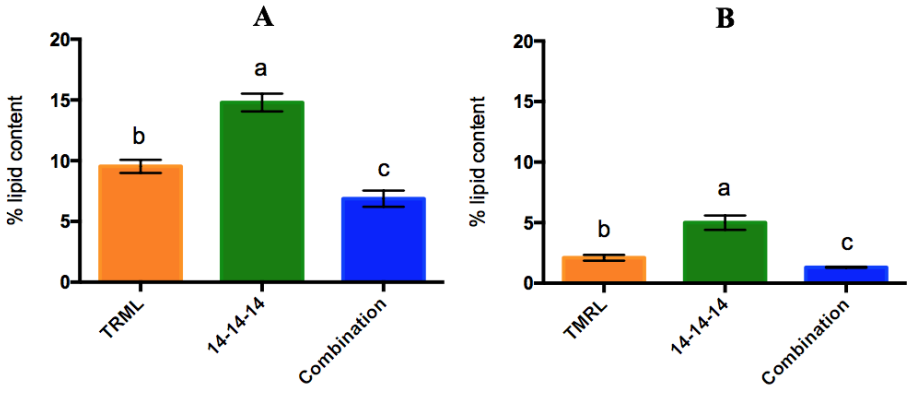

Figure 2. Average ±SEM percent dry weight lipid content of *Tetraselmis tetrahele* (A) and *Nannochloropsis* sp. (B) (n=5).

DISCUSSION

Growth Rate

Supply of nutrients plays a key role in algal growth both in artificial cultures and in the natural environment (Parrish el al. 1987; Pal et al. 2011; Khan et al. 2018). Nutrients such as nitrogen, phosphorus, and silicon are considered as periodically growth-limiting nutrients for phytoplankton in the marine environment (Tornabene et al. 1983; Parrish et al. 1987; Yu et al. 2009; Breuer et al. 2012; Recht et al. 2012). Although the effect of factors mentioned above were not studied in detail in the present experiment, the amount of nitrogen in each treatment can be estimated as lower in 14-14-14 fertilizer compared to the combination and TMRL based on their N-P-K values. The Nitrogen is 14% in 14-14-14 and 35% in combination. In TMRL, the nitrite in potassium nitrite is the source of nitrogen in the culture.

Based on the study conducted by Lim et al. (2012), among *Chaetoceros calcitrans*, *Chaetoceros muelleri*, *Isochrysis galbana*, *Nannochloropsis* sp., *Chlorella* sp., *Pavlova lutheri* and *Tetraselmis* sp., *Tetraselmis* sp. exhibited the second highest growth rate next only to *Tetraselmis chui*. In this study, our assumption is that the *T. tetrahele* cultured under combination and TMRL could have suffered from nitrogen toxicity based on the high initial amount of nitrogen in each fertilizer upon preparation of treatments thus exhibiting low growth rate compared to that grown in 14-14-14. Kim et al. (2016) mentioned

that the high growth rate in *Tetraselmis* cells is due to its ability to utilize other nitrogen and carbon sources efficiently.

Nannochloropsis sp. on the other hand exhibited good growth which can be attributed to the relatively high amount of nitrogen and other nutrients that are essential for their growth present in TMRL and not in commercial fertilizers. Ma et al. (2014) reported a specific growth rate for nine *Nannochloropsis* strains that ranged from 0.11 to 0.21 per day and this range is comparable to the results of the present study for the *Nannochloropsis* sp. cultured under TMRL.

Average Lipid content

Bligh-Dryer method, a cost-effective method for extracting lipid is normally used for extracting total lipids from microalgae (Maizatul et al. 2017). The amount of lipid extracted from microalgae varies depending on species, culture condition, nitrogen and carbon source, culture media used, and light intensity (Hu et al. 2008; Zienkiewicz et al. 2016; Alishah et al. 2019). Lipid accumulation takes place in many algae as a response to exhaustion of the nitrogen supply, several species of microalgae are found to accumulate large amounts of lipids in the cells under nitrogen limited growth (Martin et al. 2014; Suyono and Samudra 2015). This observation was relatively true for *T. tetrahele*, wherein *T. tetrahele* cultured under 14-14-14 fertilizer exhibited high growth rate and had the highest amount of lipid present. Interestingly on the other hand, in the case of *Nannochloropsis* sp. that were cultured in 14-14-14 fertilizers, low growth rate had yielded a high amount of lipid. This trend was also observed in *Nitzschia* sp. which despite its slow growth rate had a high lipid productivity (Duong et al. 2015).

In terms of lipid content, in Jena et al. (2012), 24% lipid per dry weight during the early stationary phase of *Scenedesmus* sp. was observed which was higher than the lipid content attained in the present study. Dayananda et al. (2005) also reported that the lipid content from *Botryococcus* sp. extracted using chloroform: methanol (1:2) reached 14% which was comparable to the extracted lipid content from *T. terahele* of this study but not for *Nannochloropsis* sp.

Detailed study on the lipid composition and metabolism in response to nitrogen deprivation is recommended in order to verify the difference in lipid production in microalgae. Further, strain improvement through improvement of genes could be used to enhance the lipid content of microalgae.

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